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# Genetic Diversity of *Cylindrospermopsis* Strains (Cyanobacteria) Isolated from Four Continents†

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**The genetic diversity of *Cylindrospermopsis* strains (cyanobacteria) was examined using mainly the 16S-23S internally transcribed spacer (ITS1) sequences. Strains were grouped in three clusters: (i) America, (ii) Europe, and (iii) Africa and Australia. These results suggested a recent spread of *Cylindrospermopsis* across the American and European continents from restricted warm refuge areas instead of exchanges between continents. On the other hand, they also suggested a recent colonization of Australia by African strains.**

*Cylindrospermopsis* Seenayya et Subba Raju 1972 (25) is a filamentous, heterocystous freshwater cyanobacterium genus belonging to the *Nostocales* order. The type species *Cylindrospermopsis raciborskii* was first identified in a sample collected in Java island (27) and further described as a typical tropical cyanobacterium (23). The geographical distribution of this toxic cyanobacterium has now extended from tropical to temperate areas (5–8, 17, 23). Past studies on phytoplankton diversity have indicated that *C. raciborskii* appeared in Europe during the 1930s. In addition, recent reviews (16, 23) clearly have shown a progressive colonization by *C. raciborskii* from Greece and Hungary towards Northern latitudes near the end of the 20th century. Two possible explanations of the spread of *C. raciborskii* towards temperate areas have been proposed by Padisák (23): either a primary radiation from Africa or the dispersal of adapted strains from Australia to the Eurasian and eventually American continents.

To test the hypotheses on the origin of European *C. raciborskii* strains and more globally on the phylogeography of this species, 16 *Cylindrospermopsis* strains and two *Raphidiopsis* strains isolated from freshwater lakes and reservoirs in Africa, America, Australia, and Europe were characterized genetically. The genotypic characterization was based on sequencing of the 16S-23S internally transcribed spacer (ITS1) of the ribosomal operon and completed for some of them by the sequencing of fragments of the 16S rRNA, *rpoC1*, and *nifH* genes.

The 18 strains used in this study are listed in Table 1. Based on morphological criteria, two of them were identified as *Raphidiopsis* sp. Fritsch et Rich 1929 (12). In contrast with the 16 other strains, these two strains lack the ability to differentiate heterocysts when grown in a nitrogen-free medium and to fix nitrogen, since their cultures in this condition turned from

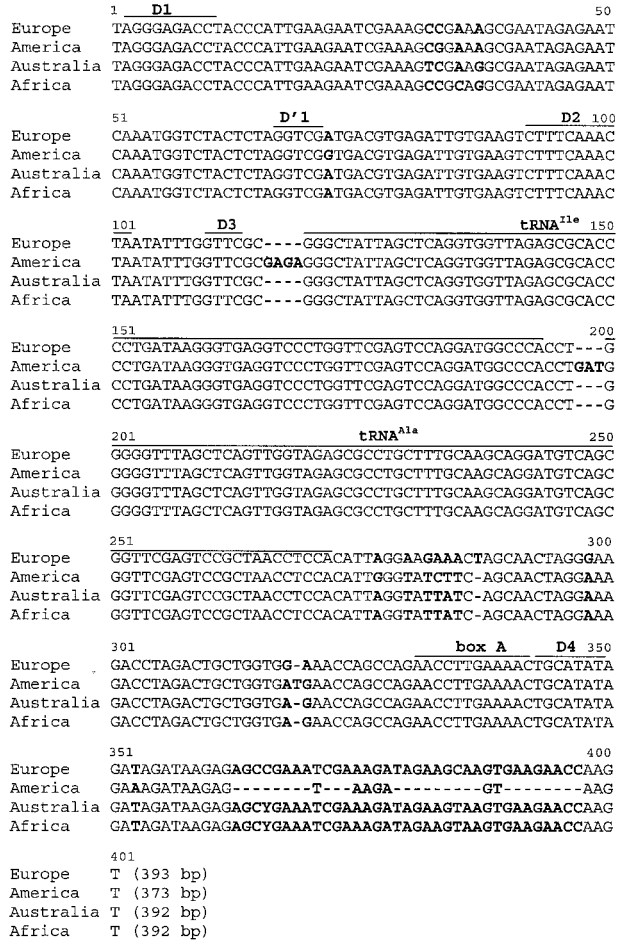


FIG. 1. Alignment of the consensus sequences obtained for *Cylindrospermopsis* and *Raphidiopsis* ITS1-L from each continent. Europe: ITS-L from six *Cylindrospermopsis* strains; America: ITS-L from six *Cylindrospermopsis*/*Raphidiopsis* strains; Australia: ITS-L from three *Cylindrospermopsis* strains; Africa: ITS-L from three *Cylindrospermopsis* strains.

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TABLE 1. Cyanobacterial strains used in this study

| Species | Strain[a] | Geographic origin | Locus sequenced[b] | | | | |
|---|---|---|---|---|---|---|---|
| | | | 16S | ITS1-S | ITS1-L | *rpoCl* | *nifH* |
| *Cylindrospermopsis raciborskii* | PMC98.14 | Viry-Châtillon, France | + | + | + | + | + |
| | PMC99.12 | Chanteraînes, France | + | + | + | + | + |
| | PMC114.02 | Courneuve, France | | | + | | + |
| | ACT-9502 | Balaton, Hungary | + | + | + | | |
| | CYLI 53 | Lake Melangsee, Germany | | | + | + | |
| | PMC99.06 | Epazote, Mexico | | | + | | + |
| | PMC99.08 | Jabali, Mexico | | | | + | + |
| | PMC00.01 | Jucazinho, Brazil | + | + | + | | |
| | ITEP-A3 | Riacho do Pau, Brazil | + | | + | | |
| | ITEP-018 | Tabocas reservoir, Brazil | + | | + | | |
| | CYP-030B | Bourke, NSW,[c] Australia | + | | + | + | |
| | CYP-023 | Bourke, NSW, Australia | | | + | | |
| | CYP-026J | Bourke, NSW, Australia | | + | + | + | |
| | PMC118.02 | Guiers, Senegal | | | + | + | + |
| | PMC117.02 | Guiers, Senegal | | | + | + | + |
| *Cylindrospermopsis africana* | PMC115.02 | Guiers, Senegal | | | + | + | + |
| *Raphidiopsis* sp. | | | | | | | |
| | ITEP-005 | Tapacurá, Brazil | + | + | + | + | NO |
| | ITEP-007 | Ingazeira, Brazil | + | + | + | | |

[a] Culture collections: PMC, Paris Museum Collection, C. Bernard of Museum National d'Histoire Naturelle, Paris, France; CYLI, J. Fastner of Technical University Berlin, Berlin, Germany; ACT, A. Kovacs of Institute of the Hungarian Academy of Sciences, Tihany, Hungary; ITEP, R. Molica of Instituto Tecnologico do Estado de Pernambuco, Recife, Brazil; CYP, P. Baker of Australian Water Quality Center, Salisbury, Australia.

[b] +, sequence obtained; NO, sequence not obtained for this isolate.

[c] NSW, New South Wales.

green to yellow within a week. However, the partial 16S rRNA gene sequences (348 bp) of the two *Raphidiopsis* strains, using the cyano-specific primers (21), were identical to those of seven *Cylindrospermopsis* strains of this study (Table 1) and of 25 *Cylindrospermopsis* strains available in the GenBank database (data not shown). The clonal isolates were grown in Z8 or Z8X (without nitrogen) medium at 25°C in Erlenmeyer flasks, illuminated at 25 μmol of photons $\cdot m^{-2} \cdot s^{-1}$ with a 16:8-h light-dark photoperiod. For the genetic analysis, 2 ml of culture in the early growth phase was centrifuged, washed once in sterile water, and frozen in liquid nitrogen. The ITS1 was amplified using the primers 322 and 340 (15), whereas the *rpoC1* and *nifH* genes were amplified using the primers *rpoC1*f (5′-ACCATTAACTACCGCACCCT-3′) and *rpoC1*r (5′-TTG TCAATTACCCGCAGACG-3′) and *nifH*f (5′-CGTAGGTT GCGACCCTAAGGCTGA-3′) and *nifH*r (5′-GCATACATC GCCATCATTTCACC-3′). All amplifications were performed in a volume of 50 μl containing 2 μl of clonal culture, 200 μM deoxynucleoside triphosphate, 20 μM (each) primer, and 5 μl of 10× PCR buffer, using the same PCR conditions as described by Gugger et al. (13). The ITS PCR products were cloned prior to sequencing, while the *rpoC1* and *nifH* PCR products were directly sequenced using the same primers as for amplification. Sequencing was performed using the Applied Biosystems 373 automated DNA sequencer (Perkin-Elmer, Foster City, Calif.) according to the manufacturer's instructions. The sequences, obtained independently for both strands, were aligned using the PILEUP module of the GCG package (Genetics Computer Group, Inc., Madison, Wis.) and edited manually with GeneDoc (20). Phylogenetic trees were constructed by using the neighbor-joining method on Jukes-Cantor pairwise distances by maximum parsimony and by maximum-likelihood analyses, using the PHYLIP software package (11).

**ITS1 rRNA analysis.** Two amplified products, ranging from 408 to 435 bp and from 569 to 593 bp, respectively, corresponding to short and long ITS1 with flanking regions, were obtained from each strain. The short (ITS1-S) and long (ITS1-L) ITS1 products were sequenced. The ITS1-L sequences contained two tRNA genes and were more informative than the ITS1-S sequences, because the flanking regions of each tRNA gene were polymorphic (Fig. 1). The ITS1-L sequences of the *Cylindrospermopsis* and *Raphidiopsis* American strains were about 20 bp shorter than the ones from other continents (Fig. 1). A very high sequence similarity (>99%) was revealed within European strains, American strains, and African and Australian strains, whereas less than 91% similarity was found between these three groups of strains. The phylogenetic analysis was consistent with these findings and identified three clusters highly supported by the bootstrap analysis, whatever the phylogenetic algorithm used (Fig. 2).

***rpoC1* and *nifH* gene analysis.** In order to compare some of our strains to *Cylindrospermopsis* sequences available in the GenBank database, a 380-bp fragment *rpoC1* gene was obtained from 10 strains originating from the four continents (Table 1). The *rpoC1* gene did not yield much information due to the low level of polymorphism (36 out of 380 positions). There was a high homology between the European and the African and Australian *Cylindrospermopsis* sequences (98 to 100% identity) and between the sequences of the *Raphidiopsis* strain from Brazil and the *Cylindrospermopsis* strain from Mexico (96% identity). The phylogenetic analysis (data not shown) allowed only the differentiation of the American strains from all the other strains. A 297-bp fragment of the *nifH* gene from three African, three French, and two Mexican *Cylindrospermopsis* strains (Table 1) was sequenced for comparison with 19 sequences of this locus available in the GenBank database

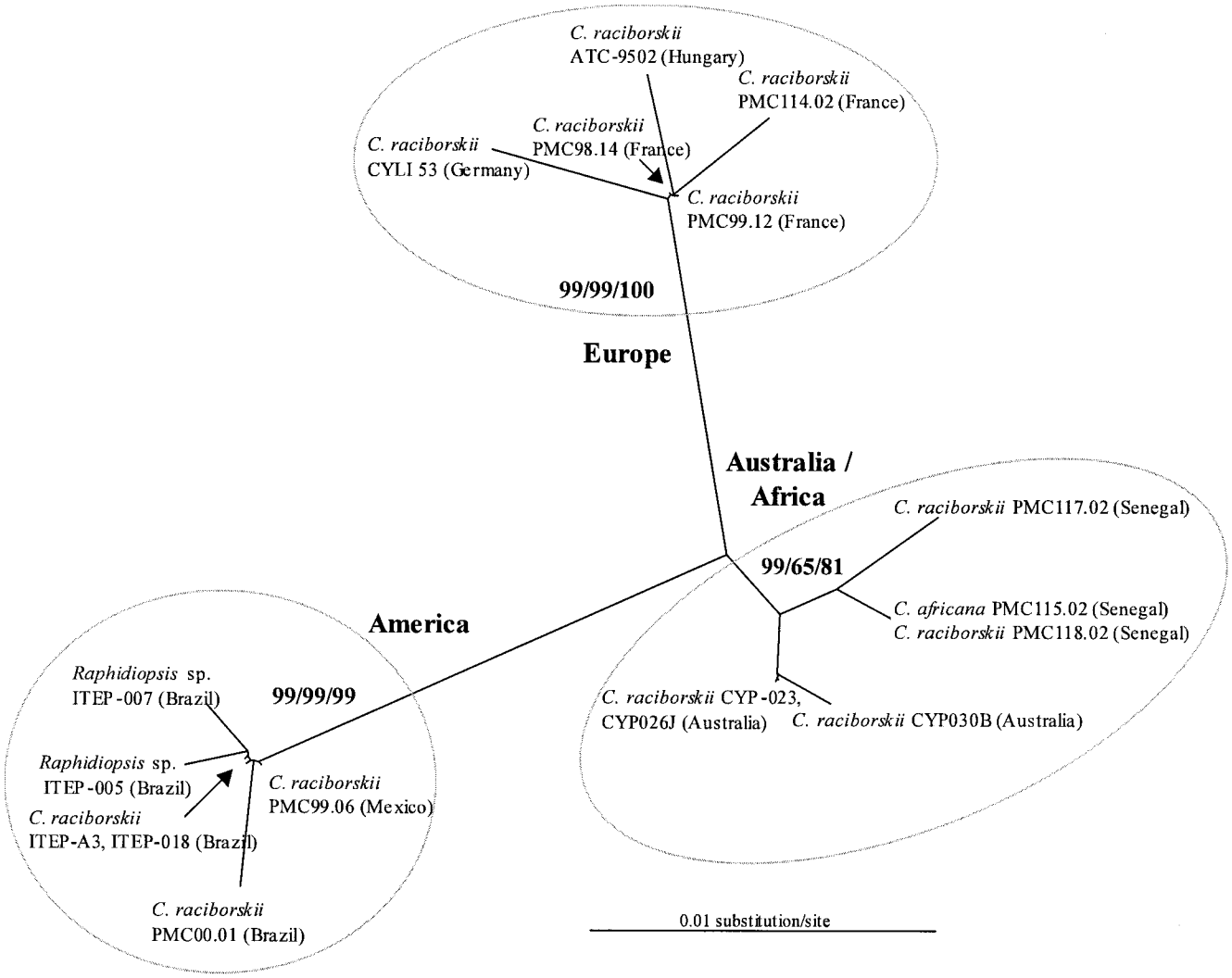


FIG. 2. Maximum-likelihood unrooted tree based on *Cylindrospermopsis* and *Raphidiopsis* ITS1-L sequences. Neighbor-joining, maximum-parsimony, and maximum-likelihood bootstrap values >70%, respectively, are given at the nodes (500 bootstrap resampling).

(10). No amplification of the *nifH* gene was obtained for the *Raphidiopsis* strains, whereas amplicons were retrieved with these primers for other nostocalean strains, such as *Anabeana* and *Aphanizomenon* strains (data not shown). In *Cylindrospermopsis* strains, this locus was highly conserved (>98% similarity). The *nifH* sequences of the 10 European strains were identical, and the 3 African sequences were similar to those of the 6 Australian strains. Finally, the eight American sequences were divergent by only five nucleotide substitutions. Four sites allowed the distinction of three groups of strains on the basis of the three continental areas: Africa and Australia, America, and Europe.

The ITS1 and *rpoC1* loci showed that *Cylindrospermopsis* and *Raphidiopsis* strains from the same continent were more closely related to each other than the *Cylindrospermopsis* strains originating from different continents. Thus, it would be very interesting to perform a more complete study using a polyphasic approach in order to investigate the question of the taxonomic validity of these two genera.

Previous studies based on 16S rRNA gene, *cpcBA*-IGS, and *nifH* sequences (10, 19) had revealed a separation of the Australian, European, and American strains. But these loci contained too few polymorphic sites to distinguish clusters supported by the bootstrap analyses. Therefore, the ITS1 fragment is, so far, the only genetic marker providing robust phylogenetic inferences between *Cylindrospermopsis* strains. This marker was previously used to study subgeneric phylogenetic relationships in *Microcystis* (14, 22), *Arthrospira* (1, 24), and *Microcoleus* (2).

Our findings with ITS1 suggest that the recent invasion of Europe and of Central and North America by *Cylindrospermopsis* did not result from recent colonization events by African or Australian isolates as proposed by Padisák (23). In view of the history of the earth's climatic changes and of biogeographic evidence for numerous plant and animal models (e.g., see references 18 and 26), we can propose the following hypothesis. First, the multiple glaciations or dry climatic conditions, during the Pleistocene age, for example, could have led to the extinction of *Cylindrospermopsis* in most of its geographical distribution areas and allowed it to survive only in warm

refuge areas on each continent. More recently, the elevation of temperatures has allowed the colonization of more and more septentrional areas from these warm refuge areas on the European and American continents. This hypothesis is in agreement with our ecophysiological study with the same strains, showing that the light and temperature optima for the growth of the European isolates are the same as those for tropical ones (3, 4) and, therefore, that these European strains could have originated in a warm area located on the Eurasian continent.

To test this alternative hypothesis on the spread of *C. raciborskii* from warm refuge areas, it would be very interesting to evaluate the phylogenetic relationship between strains from Europe and strains from such potential warm refuge areas on the Eurasian continent. For example, the Indonesian Peninsula could be examined, because in this region the climate has been consistently tropical over the past 100 million years. But other possibilities also exist, the investigation of which would require extensive sampling on the whole Eurasian continent.

Finally, concerning the close relationship between African and Australian strains, more ITS1 sequences are needed from strains isolated on each of these continents in order to confirm a recent colonization of Australia by strains originating from Africa. Since Australia has been subjected to periods of cooling during the earth's history, this could have led to the local extinction of the species in this isolated continent and to the need of a recent colonization, in relation with human activities, for example.

In conclusion, our molecular data suggest an alternative hypothesis to that based on phenotypic and ecological data to explain the actual distribution of *C. raciborskii* in the world. The use of the ITS1 molecular marker on an extensive set of strains will probably provide an answer to this question. Whatever the origin of the European strains is, the spread of *Cylindrospermopsis* on the European continent is currently advancing rapidly, as demonstrated by the increasing occurrence of this species in several water bodies in France (9).

**Nucleotide sequence accession numbers.** Nucleotide sequences have been deposited in the GenBank-EMBL database under the accession numbers AJ582102 to AJ592110 (16S rRNA gene), AJ831386 to AJ831392 (ITS1-S), AJ582268 to AJ582284 (ITS1-L), AJ582089 to AJ582093 and AJ582285 to AJ582289 (*rpoC1*), and AJ582094 to AJ582101 (*nifH*).

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